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Insect erythrocyte agglutinins. *In vitro* opsonization experiments with *Clitumnus extradentatus* and *Periplaneta americana* haemocytes

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Summary. The effect of naturally occurring haemagglutinins on the *in vitro* phagocytosis of sheep erythrocytes by the blood cells (haemocytes) of *Clitumnus extradentatus* and *Periplaneta americana* was studied. The results showed that the haemagglutinins in both species failed to act as opsonins. Indeed, in some instances, incubation of erythrocytes in haemolymph resulted in less avid ingestion as compared with the saline-incubated controls. This reduced phagocytosis was probably caused by the clumping of erythrocytes on the haemocyte monolayers, leaving fewer single red cells available for uptake. The possible roles of these erythrocyte agglutinins in the host defence systems of insects are discussed.

INTRODUCTION

Phagocytosis is an important component in the cellular defence reactions against invading microorganisms in both invertebrates and vertebrates. With vertebrate phagocytes, the attachment of particles prior to ingestion is usually dependent on immunoglobulins and complement. However, in invertebrates, true antibodies are wanting, and although primitive complement-like factors do exist (Day, Gerwurz, Johannsen, Finstad & Good, 1970; Anderson, Day & Good, 1972) their functional significance is yet to be elucidated. Many workers have, however, found mainly non-inducible humoral substances in the blood which agglutinate bacteria or erythrocytes *in vitro* (e.g. Bernheimer, 1952; Cann, 1974; Anderson & Good, 1975; Pistole, 1976, 1978; Ratcliffe & Rowley, 1979, 1980), and these substances have been partially characterized in a number of invertebrate species (Acton & Weinheimer, 1974). Some invertebrate agglutinins have been shown to act as opsonins and facilitate phagocytosis (Tripp, 1966; Tripp & Kent, 1967; Prowse & Tait, 1969; McKay & Jenkin, 1970; Paterson & Stewart, 1974; Anderson & Good, 1976; Hardy, Fletcher & Olafsen, 1977; Renwrantz & Mohr, 1978; Sminia, van der Knaap & Edelenbosch, 1979) although, occasionally, opsonic activity is apparently lacking (Fuke & Sugai, 1972; Smith & Ratcliffe, 1978).

Few studies exist into the role of agglutinins in the cellular defences of insects, and although Scott (1971) showed that in the cockroach, *Periplaneta americana*, the naturally occurring haemagglutinins did not apparently act as opsonins, the results of this study were based only on the degree of attachment of test particles since little phagocytosis was observed. More recently, Anderson, Holmes & Good (1973) reported that concentrated haemolymph from the cockroach, *Blaberus craniifer*, did not significantly stimulate the killing of a number of bacterial species following phagocytosis. The fact that such a paucity of information exists on the role of agglutinins in insects is clearly

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unsatisfactory. Therefore, in order to extend our knowledge of insect haemagglutinins, we have recently described the distribution of these substances in the cell-free haemolymph (blood) of a number of insect species and found that the level of activity not only differed markedly from species to species but was also mainly non-inducible (Ratcliffe & Rowley, 1980). In the present study, we have attempted to determine whether the haemagglutinins of the stick insect, *Clitumnus extradentatus*, and the cockroach, *Periplaneta americana*, act as opsonins. These two species were chosen as experimental animals because of their relatively high haemagglutination titres (Ratcliffe & Rowley, 1980) and their large numbers of freely circulating blood cells, which facilitate such *in vitro* studies.

MATERIALS AND METHODS

Insects

Adult female *C. extradentatus* and adult male *P. americana* were used in the present study, and fed on fresh bramble leaves or rat pellets, respectively. All insects were from healthy stocks which had been raised in an insectary at $25 \pm 3^{\circ}$ for at least 2 years.

Haemocytes

Single insects were used in each experiment. They were placed on ice for 10–15 min to slow down haemolymph coagulation and to facilitate bleeding (Ratcliffe & Rowley, 1975; Rowley & Ratcliffe, 1976). A walking leg was then amputated at the coxa and the exuding blood quickly collected, diluted, and thoroughly mixed in a syringe containing 0·2–0·3 ml of ice-cold Carlson's saline. The contents of the syringe were immediately ejected into a further 1 ml of ice-cold saline and the cell number counted and adjusted prior to monolayer formation (see below).

Serum

Bleeding was carried out as above, except that in *P. americana* the blood was rapidly diluted in a Thoma white blood cell pipette with ice-cold Carlson's saline to give a known dilution. In *C. extradentatus*, however, no dilution was necessary due to the large volume of blood available. The cells and any coagula were removed by centrifugation at 1200 *g* for 10–15 min and the serum tested for its haemagglutinating (HA) activity. Samples from individual insects of known titre were stored at -20° for up to 6 months before use. Such storage had no apparent effect on the HA activity.

Determination of HA activity

HA activity of the serum samples, prior to their use in the opsonization experiments, was determined as described previously (Ratcliffe & Rowley, 1980). Serum was serially diluted against 0·85% saline and then an equal volume of a 2% solution of formalized sheep erythrocytes (FSRBC; Difco Laboratories, England) was added. Results were expressed as reciprocal values of the last serum dilution that clearly agglutinated the erythrocyte suspensions.

Erythrocyte opsonization procedure

FSRBC were washed four to five times in Carlson's saline and adjusted to approximately $2{\cdot}5 \times 10^7$ FSRBC/ml. The suspension was divided into two equal aliquots and centrifuged at 300 *g* for 5–10 min. One aliquot was resuspended in an equal volume of serum of a known HA titre (see below) and the other in the same volume of saline. Both tubes were then incubated for 45 min at 26° on a roller drum.

Preliminary experiments indicated that FSRBC incubated in serum at a greater concentration than the HA endpoint (*P. americana* = 8—160; *C. extradentatus* = 512–2048, see Ratcliffe & Rowley, 1980) were strongly agglutinated during the incubation period. In an attempt to overcome this problem, the FSRBC were incubated in diluted serum at concentrations close to the endpoint. Thus, in *P. americana* the FSRBC were incubated in serum at half above or one half below the endpoint. Pooled serum was used to give a sufficient volume for 'opsonization'. However, in *C. extradentatus* three serum dilutions (128, 1024 and 2048) were prepared from a 'standard' sample from one insect with a titre of 1024.

Despite these precautions, in both *P. americana* and *C. extradentatus*, a certain amount of agglutination occurred during 'opsonization' of the FSRBC. The clumps of red cells formed, however, were broken up into single cell suspensions by ejection through a 23 gauge needle. The suspensions were checked microscopically before use to ensure that they contained only single erythrocytes. Saline-incubated erythrocytes were similarly treated even though no distinct agglutination occurred during incubation.

Finally, as a precaution against FSRBC clumping on the haemocyte monolayers during the phagocytosis experiments (see below), half of the serum-incubated erythrocytes were sedimented and resuspended as a single cell suspension in an equal volume of Carlson's saline. Thus, at each serum dilution three erythrocyte aliquots were available for incubation with the mono-

layers; serum-incubated and unwashed (SI), serum-incubated and washed (SIW), and saline-incubated controls(C).

Incubation of haemocytes and FSRBC

Diluted haemolymph containing 5×10^4 haemocytes was pipetted onto each tissue culture-washed glass coverslip and left to settle and attach for 15 min at 26°. The coverslips were then carefully rinsed with Carlson's saline, to remove non-adherent cells and all traces of haemolymph. Whenever possible, six monolayers were set up from each insect, and duplicates were overlaid with 0·1 ml of (a), serum-incubated and unwashed (SI) FSRBC, (b) serum-incubated and washed (SIW) FSRBC, or (c) saline-incubated control (C) FSRBC.

The monolayers were incubated at 26° in a moist atmosphere for 60 min, vigorously rinsed in Carlson's saline to remove non-ingested FSRBC, fixed for 10–20 min in either formalin vapour or 2·5% glutaraldehyde in cacodylate buffer (Sabatini, Bensch & Barnett, 1963), rinsed, and examined with a Leitz SM-Lux microscope with a Zeiss Neofluar × 100 phase objective. The non-adherent FSRBC, were later examined to discover if any clumps had formed during monolayer incubation.

To determine the phagocytic indices (i.e. the percentage of haemocytes containing one or more erythrocytes), 250–500 morphologically intact haemocytes were examined in random fields on each monolayer and a mean value calculated. The percentage of phagocytic haemocytes containing 1–3, 4–6, 7–9 and 10+ intracellular FSRBC was also calculated and the mean number of FSRBC per 100 haemocytes derived from these values.

Data analysis

Levels of significance of differences of means were determined using the Student's *t* test. The level of significance was $P \leqslant 0{\cdot}05$.

RESULTS

Despite the attempts to prevent erythrocyte agglutination on the monolayers (see 'Erythrocyte opsonization procedure', above), with FSRBC opsonised in the sera with higher HA concentrations (i.e. one half above endpoint for *P. americana*, 128 and 1024 for *C. extradentatus*), erythrocyte clumping tended to occur in both the SI and SIW preparations. With sera of lower HA activity, however, (i.e. one half below endpoint for *P. americana* and 2048 for *C. extradentatus*), erythrocyte clumping was reduced by washing the opsonized erythrocytes before placing on the monolayers (=SIW preparations). Despite this washing procedure, there was evidence that the SIW erythrocytes were effectively coated with serum factors since they formed a veil of agglutinated particles if left to precipitate in the opsonization tubes.

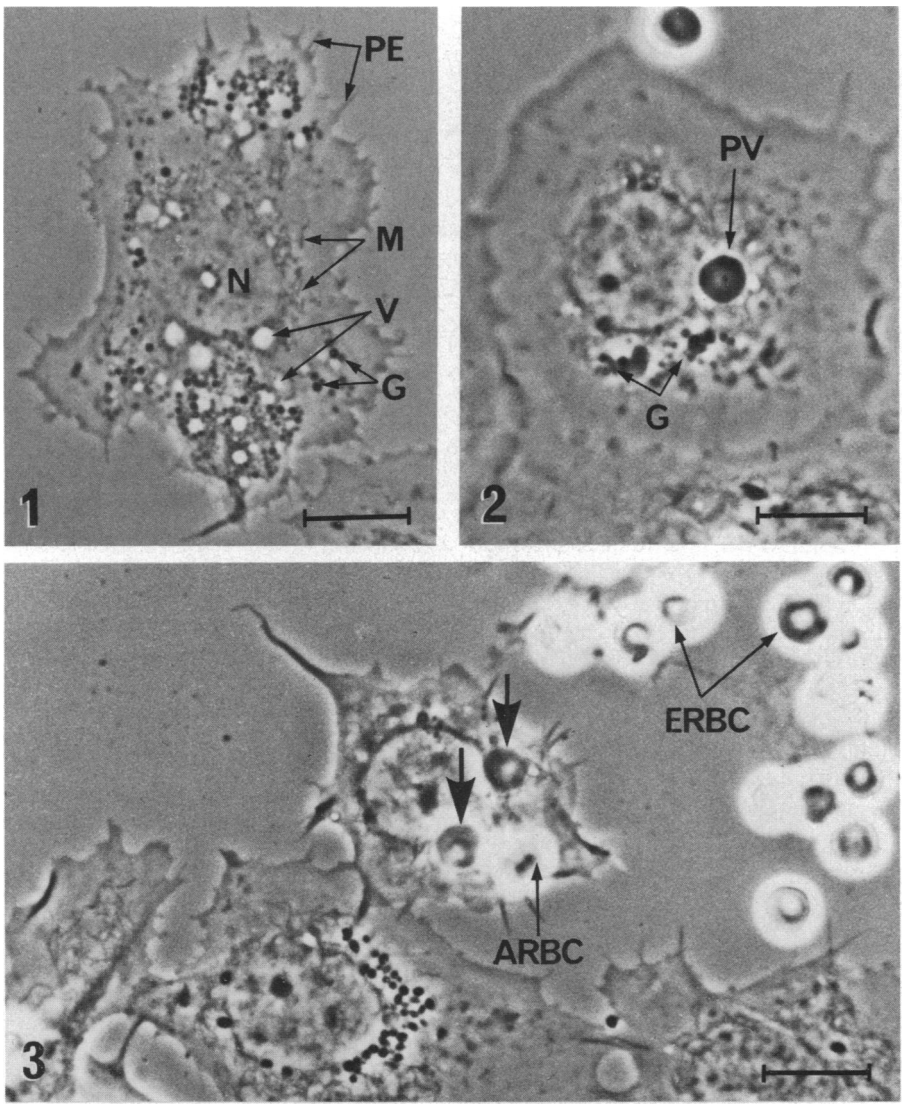
Opsonization experiments

The results of these experiments are considered separately for the two species studied.

Periplaneta americana. Five types of blood cells (haemocytes) were found in the haemolymph of *P. americana* and using the classification scheme of Price & Ratcliffe (1974) these were identified as prohaemocytes, plasmatocytes, granular cells, spherule cells and cystocytes. The plasmatocytes are the main phagocytic cell type and were the only cells to attach to the coverslips to form monolayers. After 60 min incubation, these cells were well spread-out and firmly attached to the glass. Morphologically, they resembled vertebrate macrophages with a cytoplasm containing prominent phase-dark granules, vacuoles and mitochondria, and a cell periphery characterized by ruffled membranes and spike-like protoplasmic extensions (Fig. 1). *In vitro*, these cells slowly released their granules (compare Fig. 1 with Figs 2 and 3) (Ratcliffe & Rowley, 1979); a process which superficially resembles vertebrate macrophage and neutrophil degranulation (Hirsch, 1962; Davies, Page & Allison, 1974).

Intracellular FSRBC although rarely surrounded by distinct phagocytic vacuoles (Figs 2 and 3) were easily distinguished from extracellular forms by a number of criteria. First, intracellular erythrocytes had a marked loss in their refractivity as compared with extracellular forms (Fig. 3). Second, the level of the plane of focus of intracellular erythrocytes and the cytoplasmic contents was similar and third, intracellular FSRBC were often found adjacent to the nucleus which they occasionally indented. Furthermore, due to the extensive spreading of the plasmatocytes, intracellular FSRBC were usually separated by distinct regions of cytoplasm making their identification easier.

The counts showed that for *P. americana*, incubation of FSRBC in serum did not significantly increase the uptake of these particles (Fig. 4). Indeed, in



Figures 1–3. Phase contrast micrographs of the plasmatocytes of *Periplaneta americana* in monolayer culture. Scale bars, 10 μm. (1) Fully spread plasmatocyte after 15 min *in vitro* showing the cytoplasm containing many granules (G), vacuoles (V) and mitochondria (M). The cell is bordered by protoplasmic extensions (PE). Nucleus (N). (2) Plasmatocyte after approximately 75 min *in vitro* with an intracellular erythrocyte which is surrounded by a distinct phagocytic vacuole (PV). Note the small number of granules (G) present compared with the cell in Fig. 1. (3) Showing the difference in refractivity of extracellular attached (ARBC) and free (ERBC) erythrocytes in comparison with the intracellular forms (unlabelled arrows).

those monolayers overlaid with FSRBC incubated previously in serum (SI and SIW) at one half above the endpoint, a significant reduction ($P < 0{\cdot}01$ for SI, $P < 0{\cdot}02$ for SIW) in the phagocytic indices occurred as compared with the saline controls (Fig. 4). However, using serum at the lower concentration (one half below endpoint), a significant reduction ($P < 0{\cdot}002$) occurred in the phagocytic index only in those cells overlaid with erythrocytes incubated in serum and left unwashed (SI). The reduced phagocytic indices following serum incubation, in some cases, were probably caused by FSRBC clumping on the monolayers leav-

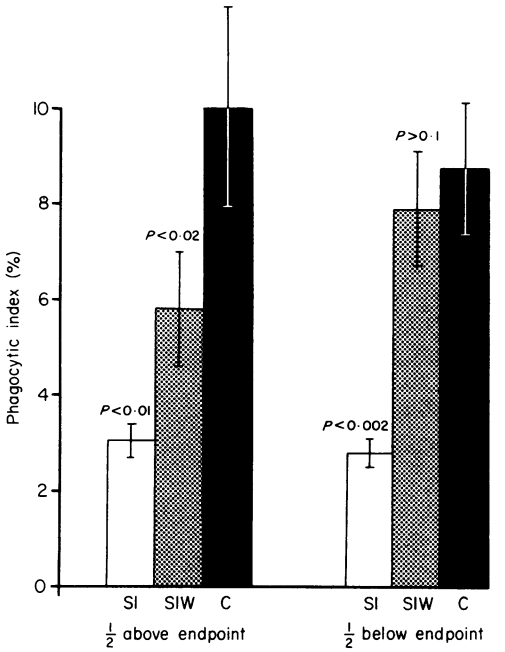


Figure 4. Phagocytic indices of the haemocytes of *P. americana* overlaid with serum-incubated and unwashed (SI), serum-incubated and washed (SIW) or saline-incubated (C) formalized erythrocytes. Mean values ± 1 SE ($n = 10$).

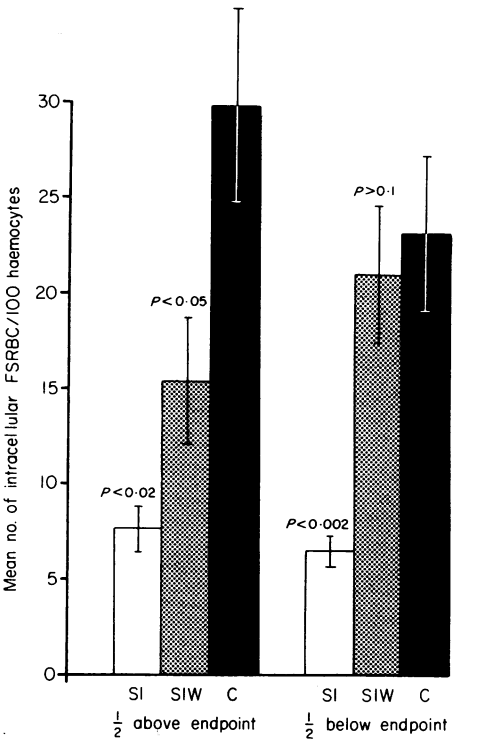


Figure 5. Showing the effect of serum incubation on the mean number of formalized erythrocytes phagocytosed/100 haemocytes in *P. americana*. SI = serum-incubated and unwashed; SIW = serum-incubated and washed, and C = saline-incubated (control) erythrocytes. Mean values ± 1 SE ($n = 10$).

ing fewer free particles available for ingestion (see Discussion).

Similar results were also found when the number of FSRBC/100 haemocytes was calculated (Fig. 5). For example, a significant ($P < 0{\cdot}02$ at one half above, $P < 0{\cdot}002$ at one half below) reduction in the numbers of FSRBC/100 haemocytes occurred in comparison to the controls, when the serum-incubated erythrocytes were left unwashed (SI).

Analysis of the results for the percentage of phagocytic haemocytes containing 1–3, 4–6, 7–9 and 10+ intracellular FSRBC showed that serum incubation apparently affected the phagocytic potential of these cells (Table 1). For example, monolayers overlaid with serum-incubated erythrocytes (one half above endpoint) showed a slight but consistent reduction in the uptake of large numbers of erythrocytes (e.g. 10+) and a marked increase in the phagocytosis of small numbers (e.g. 1–3). These trends, however, were not as marked with the lower HA concentrations (Table 1).

Clitumnus extradentatus. Five different haemocyte types have previously been described in *C. extradentatus*, namely, the prohaemocytes, plasmatocytes, granular cells, spherule cells and cystocytes (Rowley, 1977). As in *P. americana*, the haemocyte monolayers were mainly composed of flattened granule-containing plasmatocytes (Fig. 6), although, occasionally, lysed highly degenerated granular cells and cystocytes were also seen. Only the plasmatocytes ingested the FSRBC (Fig. 7), and such particles had a similar appearance to the erythrocytes within the haemocytes of *P. americana*.

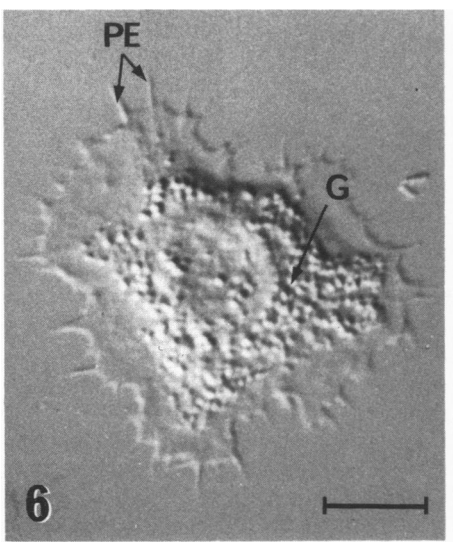
In *C. extradentatus*, as in *P. americana*, incubation in serum did not stimulate phagocytosis of the FSRBC (Figs 8 and 9) and the phagocytic indices were reduced in haemocytes challenged with serum-incubated test particles (Fig. 8). However, in comparison to *P. americana*, these reductions were less marked. For example, only in those cases where the FSRBC were incubated in serum and left unwashed (SI) were there significant reductions ($P < 0{\cdot}05$ at 1024, $P < 0{\cdot}01$ at 2048) as compared with the controls(C).

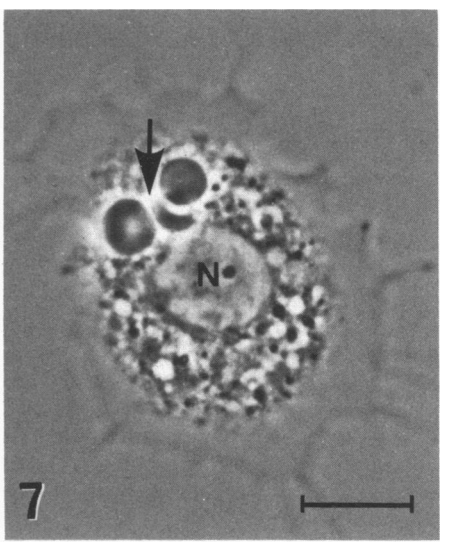
Table 1. The effect of serum incubation on the number of ingested erythrocytes/phagocytic haemocyte in *Periplaneta americana*

FSRBC* treatment	Percentage of phagocytic haemocytes containing the following number of intracellular FSRBC			
	1–3	4–6	7–9	10+
½ above endpoint				
SI	88·8±3·1†	8·9±2·4	2·3±1·0	—
SIW	81·5±2·9	13·1±2·4	5·2±1·6	0·2±0·2
C	82·6±3·2	13·3±2·1	3·3±1·1	0·8±0·5
½ below endpoint				
SI	92·0±1·7	6·9±1·7	1·1±0·7	—
SIW	85·6±2·8	11·9±2·1	2·5±0·9	—
C	85·0±2·6	12·0±1·8	2·6±0·9	0·4±0·3

* SI = serum incubated and unwashed, SIW = serum incubated and washed and C = saline-incubated erythrocytes.

† Mean values ±1 SE ($n = 10$).





Figures 6–7. Micrographs of the plasmatocytes of *Clitumnus extradentatus* in monolayer culture. Scale bars, 10 μm. (6) Nomarski interference micrograph of a typical plasmatocyte with many granules (G) and protoplasmic extensions (PE). (7) Phase contrast micrograph showing the appearance of three intracellular erythrocytes (unlabelled arrow) in the cytoplasm adjacent to the nucleus (N).

Incubation in serum had no significant effect on the number of FSRBC ingested per 100 haemocytes (Fig. 9). There was, however, some reduction in the values for the serum incubated erythrocytes as compared with the controls but the large variation indicated by the high standard errors probably accounted for the lack of significance.

Analysis of the results for the percentage of phagocytic haemocytes containing 1–3, 4–6, 7–9 and 10+ intracellular FSRBC showed that incubation in serum with a HA titre of 128 caused a significant increase ($P < 0.05$) in the number of cells containing 10+ erythrocytes as compared with the controls (Table 2). Conversely, after serum incubation, the number of cells containing 1–3 FSRBC was reduced as compared with the controls (Table 2). With the lower HA con-

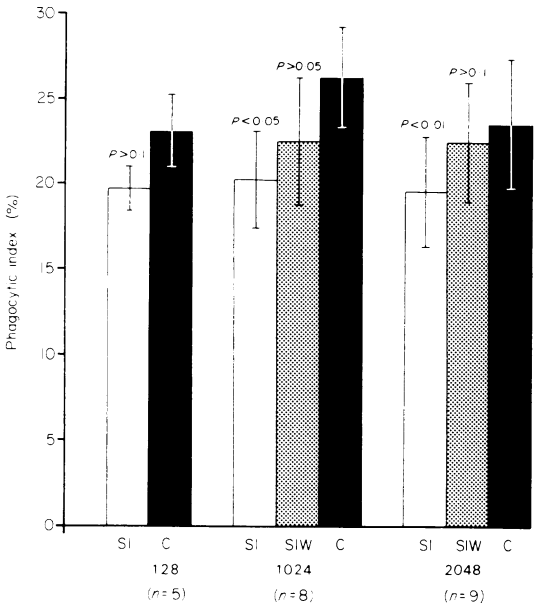


Figure 8. Phagocytic indices of the haemocytes of *C. extradentatus* overlaid with serum-incubated and unwashed (SI), serum-incubated and washed (SIW) or saline-incubated (C) formalized erythrocytes. Mean values ± 1 SE.

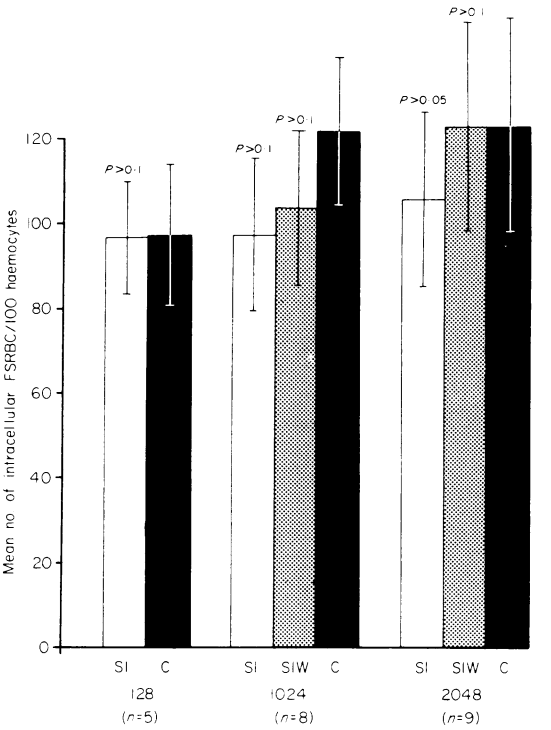


Figure 9. Showing the effect of serum incubation on the mean number of formalized erythrocytes phagocytosed 100 haemocytes in *C. extradentatus*. SI = serum-incubated and unwashed; SIW = serum-incubated and washed, and C = saline-incubated (control) erythrocytes. Mean values ± 1 SE.

centrations of 1024 and 2048 no significant differences were apparent between any of the groups, although the percentage of phagocytic haemocytes containing 10+ erythrocytes after serum incubation tended to be higher.

DISCUSSION

The results of the present paper indicate that the naturally occurring haemagglutinins of *C. extradentatus* and *P. americana* do not act as opsonins. This agrees with the earlier reports of Scott (1971) and Anderson *et al.* (1973) and indicates that in contrast to some other invertebrates (see Tripp, 1966; Prowse & Tait, 1969) that in insects these substances do not enhance phagocytosis of sheep erythrocytes. Perhaps in insects, as suggested by Tyson, McKay & Jenkin (1974), the recognition factors are in some way bound to the membrane of the phagocytes so that few remain free in the haemolymph.

In the two insect species tested, incubation of erythrocytes in serum often reduced the phagocytic indices as compared with the saline controls. This phenomenon was probably caused to some extent by clumping of the erythrocytes on the monolayers, leaving fewer single particles available for ingestion. Evidence in support of this hypothesis was obtained by washing the serum-incubated erythrocytes since this minimized the clumping reaction and in turn decreased the reduction in the phagocytic indices, which almost became identical to the saline controls (see Figs 4 and 8).

In *C. extradentatus*, erythrocyte clumping on the monolayers was probably also responsible for the increase in the percentage of phagocytic haemocytes containing groups of 10+ serum-incubated erythrocytes (HA dilution 128). These FSRBC were probably ingested as single large masses, indicating the great phagocytic potential of these cells. Concomitantly, fewer single erythrocytes would have been present on the monolayers and this probably resulted in the slight reduction in the number of haemocytes containing between 1 and 3 test particles. In *P. americana*, however, different trends existed and serum incubation caused a reduction in the number of phagocytic

Table 2. The effect of serum incubation on the number of ingested erythrocytes/phagocytic haemocytes in *Clitumnus extradentatus*

FSRBC* Treatment	Percentage of phagocytic haemocytes containing the following numbers of intracellular FSRBC			
	1–3	4–6	7–9	10+
HA dilution 128‡				
SI	44·3 ± 4·7†	24·8 ± 3·9	16·8 ± 2·2	14·1 ± 3·6
C	52·5 ± 5·3	27·9 ± 1·7	16·4 ± 3·4	3·1 ± 1·0
HA dilution 1024§				
SI	47·3 ± 3·5	27·4 ± 2·5	14·2 ± 2·0	11·2 ± 2·8
SIW	45·6 ± 2·9	31·8 ± 2·5	15·5 ± 1·4	7·1 ± 1·5
C	49·7 ± 4·8	27·3 ± 1·1	14·1 ± 2·7	8·9 ± 2·3
HA dilution 2048¶				
SI	34·2 ± 3·8	33·2 ± 2·4	22·0 ± 1·9	10·7 ± 1·6
SIW	31·0 ± 3·7	35·9 ± 1·9	23·9 ± 2·5	9·2 ± 2·2
C	37·0 ± 4·0	32·3 ± 2·4	21·2 ± 2·6	10·4 ± 2·6

* SI = serum-incubated and unwashed, SIW = serum-incubated and washed and C = saline-incubated erythrocytes.
† Mean values ± 1 SE
‡ $n = 5$.
§ $n = 8$.
¶ $n = 9$.

haemocytes containing 10+ (Table 1, HA dilution one half above end-point, unwashed) and a slight increase in those containing 1–3 erythrocytes. A likely explanation for these differences is that in *P. americana* the plasmatocytes have a more limited phagocytic capacity than in *C. extradentatus* (compare Figs 4 with 8, and 5 with 9) and as a result they mainly phagocytose small aggregates of 1–3 erythrocytes rather than larger groups of FSRBC (Table 1 and 2). Clumping simply reduces the number of these single or small groups of erythrocytes available for ingestion.

Similar clumping reactions probably caused the reduction in the numbers of erythrocytes attached to the haemocytes of *P. americana* reported in the experiments of Scott (1971). Furthermore, Anderson & Good (1976) also noted that test particles clumped in high concentration of agglutinin, and this caused an apparent decrease in the rate of erythrocyte ingestion by the blood cells of the mollusc, *Otala lactea*. This reduction was abolished by further dilution of the agglutinin.

These results clearly illustrate that care must be taken in interpreting such *in vitro* experiments, since agglutination can change the number of test particles ingested and could conceivably elevate the total phagocytosis (see Table 2, 128 dilution) so falsely indicating the presence of opsonins in the haemolymph.

What other role(s) the erythrocyte agglutinins might play in insect defence reactions is unknown. However, the closely related bacterial agglutinins present in some insects (Briggs, 1958; Feir & Watz, 1964; Gilliam & Jeter, 1970) may help in clearing the haemocoele of invading micro-organisms by rapid agglutination, thus leaving large masses for phagocytosis and the process of nodule formation, in which large aggregates of micro-organisms are walled-off from the rest of the insect by a multi-layered sheath of haemocytes (Ratcliffe & Gagen, 1976, 1977). In other invertebrates, many suggestions have been put forward as to possible roles for the agglutinins present. For example, Gold, Phelps, Khalap & Balding (1974) claimed that in the sponge, *Axinella*, agglutinins were probably involved in the re-aggregation of the cells, while Cushing, McNeely & Tripp (1969) suggested that the agglutinins in some sipunculids had 'parasite immobilizing' activity. Perhaps one of the most conclusive studies of the role of agglutinins in invertebrate immunity comes from the work of Schmid (1975) with the snail, *Viviparus malleatus*. She found that the blood cells were chemotactically attracted to *Staphylococcus aureus*, but only in the presence of haemolymph agglutinins

active against this bacterium. These substances may possibly have similar functions in insects, although they may also play some non-immunological role in the life of the insect.

Finally, whether these invertebrate haemagglutinins are the fore-runners of vertebrate immunoglobulins is still uncertain. More likely, they are a separate group of substances developed in invertebrates to aid in the recognition of self/non-self and which were later replaced with the development of lymphatic systems capable of producing antibodies. Support for this hypothesis can be seen in some lower vertebrates such as lampreys (Marchalonis & Edelman, 1968) and sharks (Harisdangkul, Kabat, McDonough & Sigel, 1972) in which both haemagglutinins and chemically distinct immunoglobulins are present. Certainly, until more is known about the distribution, sites of production and quasi-immunological roles of agglutinins, particularly in 'higher' invertebrates such as tunicates and hemichordates, few definite conclusions can be made.

ACKNOWLEDGMENTS

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